Proliferating Reactive Astrocytes Are Regulated by Notch-1 in the Peri-Infarct Area After Stroke

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Background and Purpose—The formation of reactive astrocytes is common after central nervous system injuries such as stroke. However, the signaling pathway(s) that control astrocyte formation and functions are poorly defined. We assessed the effects of Notch 1 signaling in peri-infarct-reactive astrocytes after stroke.

Methods—We examined reactive astrocyte formation in the peri-infarct area 3 days after distal middle cerebral artery occlusion with or without γ-secretase inhibitor treatment. To directly study the effects of inhibiting a γ-secretase cleavage target in reactive astrocytes, we generated glial fibrillary acidic protein-CreERTM:Notch 1 conditional knockout mice.

Results—Gamma-secretase inhibitor treatment after stroke decreased the number of proliferative glial fibrillary acidic protein-positive reactive astrocytes and RC2-positive reactive astrocytes directly adjacent to the infarct core. The decrease in reactive astrocytes correlated with an increase in number of CD45-positive cells that invaded into the peri-infarct area. To study the influence of reactive astrocytes on immune cell invasion, ex vivo immune cell invasion assays were performed. We found that a γ-secretase-mediated pathway in astrocytes affected Jurkat cell invasion. After tamoxifen treatment, glial fibrillary acidic protein-CreERTM:Notch 1 conditional knockout mice had a significantly decreased number of proliferating reactive astrocytes and RC2-positive reactive astrocytes. Tamoxifen treatment also led to an increased number of CD45-positive cells that invaded the peri-infarct area.

Conclusions—Our results demonstrate that proliferating and RC2-positive reactive astrocytes are regulated by Notch 1.

Key Words: animal models • basic science • brain • brain infarction • brain ischemia • cerebral infarct • focal ischemia • gene regulation • glial cells • gliosis • ischemia

After brain injury, reactive astrocytes participate in the formation of a "glial scar." Because the glial scar has been shown to inhibit axonal regeneration, reactive astrocytes have historically been considered as a barrier to neuronal repair.1,2 However, this negative view of reactive astrocytes is changing because they have been shown to play several important roles in preserving neural tissue during the early phase of brain injuries.2–4 Knockout mice lacking two intermediate filament proteins commonly expressed by astrocytes, glial fibrillary acidic protein (GFAP), and vimentin exhibited attenuated reactive astrocyte formation, reduced glutamate transport, and increased infarct volumes 7 days after stroke.5 Reactive astrocytes were also shown to limit cellular degeneration by maintaining and repairing the blood–brain barrier and decreasing immune cell infiltration after stab injury.2,6

The emergence of reactive astrocytes in the peri-infarct area is one of the most obvious events in the brain after stroke. However, the signaling pathway(s) that control astrocyte formation and their functions in the peri-infarct area are poorly defined. Gamma-secretase-targeted proteins such as Notch 1 and APP are expressed by reactive astrocytes after brain injury.7,8 Here we determine the effects of Notch 1 signaling on a unique subpopulation of proliferative reactive astrocytes that localize immediately adjacent to the infarct core and that regulate the peri-infarct area after stroke.

Methods

Mouse

All animal work was approved by the University of Vermont College of Medicine’s Office of Animal Care in accordance with American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. Focal cerebral ischemia was produced by permanently occluding the middle cerebral artery.9–11

Full methods are available online (http://stroke.ahajournals.org).

Results

RC2-Positive Reactive Astrocytes Express Gamma-Secretase Cleavage Products

To study reactive astrocytes in the brain after stroke, we performed distal middle cerebral artery occlusion in wild type
C57b6J mice (Taconic Farms, Hudson, NY). Reactive astrocytes expressed GFAP and/or RC2 in the cortical peri-infarct area after stroke (Figure 1A). We previously identified an RC2-positive subpopulation of reactive astrocytes that formed on the ipsilateral but not the contralateral side of the brain after stroke.11 At 3 days after stroke, the number of RC2-positive cells was significantly higher in the inner cortical layer adjacent to the infarct (200 μm radially from the infarct area) compared with the outer cortical layer (200 μm radially from the inner layer; n=3, P<0.05; Figure 1B). Similarly, the number of GFAP/RC2-positive cells was significantly greater in the inner cortical layer compared with the outer layer, demonstrating that RC2-positive reactive astrocytes appear directly adjacent to the infarct core after stroke (n=3, P<0.05; Figure 1B). Ki67/GFAP-positive proliferating reactive astrocytes were also observed adjacent to the infarct core after stroke (Figure 1C). The 3 day time point was chosen because the RC2 antigen and GFAP were highly expressed at this time relative to 1 day after stroke.

Gamma-secretase activity is known to increase in the brain early after stroke.7,12 We examined the presence of γ-secretase cleavage products: APP Intracellular Domain (AICD), and Notch 1 Intracellular Domain (NICD1) in the peri-infarct area 3 days after stroke. By immunohistochemistry, GFAP-positive reactive astrocytes and RC2-positive reactive astrocytes both expressed AICD and NICD1 in the peri-infarct area (Figure 2A). We also observed expression and nuclear localization of AICD and NICD1 in neuronal nuclei (NeuN)-positive neurons (data not shown). These data suggested that γ-secretase cleavage products may regulate reactive astrocytes and RC2-positive reactive astrocytes in the peri-infarct area after stroke.

Formation of Reactive Astrocytes Is Disrupted by Gamma-Secretase Inhibitor Treatment

To determine whether γ-secretase-mediated events controlled the formation of reactive astrocytes, we inhibited Type I intracellular membrane protein cleavage with the γ-secretase inhibitor, dibenzazepine (GSI; Figure 2B). In accordance with altered γ-secretase activity early after stroke, we observed increased AICD expression levels in the peri-infarct area 1 day after stroke compared with AICD levels in sham-operated brains (n=6, P<0.01; Figure 2C). After GSI treatment, we observed decreased levels of AICD expression in the peri-infarct area compared with dimethyl sulfoxide (DMSO) treatment (n=6, P<0.05; Figure 2C). Overall NICD1 expression levels in the peri-infarct area did not increase after stroke compared with levels in sham animals. We did, however, observe a trend of decreased NICD1 expression levels in the peri-infarct area after GSI treatment compared with DMSO treatment (sham, 106%±18.9%; DMSO, 100%±15.4%; GSI, 79%±7.4%; mean±SEM, n=4 to 6 mice for each group). These data indicated that GSI treatment affected cleavage of Type I intramembrane proteins in the peri-infarct area after stroke. As expected, GFAP protein expression levels were increased in the peri-infarct area 3 days after stroke compared with sham-operated brains. In contrast, GFAP protein expression levels were decreased 3 days after stroke and GSI treatment compared with DMSO treatment (n=10 to 12, P<0.01; Figure 2D).

We quantified the number of GFAP-positive reactive astrocytes after stroke and GSI treatment. The number of GFAP/Ki67-positive proliferating reactive astrocytes was significantly decreased by GSI treatment (n=4, P<0.05; Figure 3A). We quantified also the number of RC2-positive
reactive astrocytes in the peri-infarct area after stroke and DMSO or GSI treatment. We observed a decreased number of RC2-positive reactive astrocytes in the cortical peri-infarct area after GSI treatment compared with vehicle treatment (n/H11005 3, P/H11021 0.05; Figure 3B). These data indicated that -secretase cleavage products play an important role in regulating the proliferating reactive astrocytes and the sub-population of RC2-positive reactive astrocytes in the peri-infarct area after stroke. Notably, we did not observe a significant difference in the total numbers of GFAP-positive cells after stroke with GSI treatment compared with vehicle controls (DMSO, 5255.1/H11006 437.9 cells/mm2; GSI, 5199.8/H11006 231.8 cells/mm2; mean/H11006 SEM, n/H11005 4 mice for each group, P/H1105 0.91).

To examine whether dimethyl sulfoxide or GSI treatment affected reactive astrocyte apoptosis/necrosis after stroke, we performed terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assays for mice treated with phosphate-buffered saline, DMSO, or GSI. We observed many terminal deoxynucleotidyltransferase-mediated dUTP nick end labeled (TUNEL)-positive cells in the infarct areas of mice treated with phosphate-buffered saline, DMSO, and GSI (data not shown). However, we did not observe TUNEL-positive/GFAP-positive reactive astrocytes in the peri-infarct area 3 days after stroke (data not shown).

Reduced Number of Proliferating and RC2-Positive Reactive Astrocytes and Immune Cell Invasion Into the Peri-Infarct Area

One possible role of reactive astrocytes is to prevent immune cell invasion as reported in spinal cord injury and brain stab injury.\textsuperscript{13,14} To determine whether this was the case in our model, we quantified CD45-positive monocytes at the border of the infarct region with or without GSI treatment. Because monocytes and microglia express similar proteins such as CD45 and CD11b, it is technically challenging to distinguish monocytes and microglia in the peri-infarct area. Therefore, we used CD45 as a marker and distinguished monocytes by their round morphology in the peri-infarct area. We observed a significant increase in the number of CD45-positive cells in the peri-infarct area after GSI treatment compared with the number in vehicle-treated controls (n/H11005 4 to 5, P/H1105 0.05; Figure 3C).

To study the effects of astrocytes on immune cell invasion, we isolated astrocytes from neonatal mouse brains. When cultured in serum-containing medium, the astrocytes were activated and expressed GFAP, Nestin, and RC2, similar to protein expression patterns of RC2-positive reactive astrocytes after stroke (Figure 4A). Jurkat cells (T cell line) are commonly used to study immune cell invasion.\textsuperscript{15} Jurkat cell invasion was determined with either 5% fetal bovine serum or 5% fetal bovine serum with astrocytes in the bottom wells of invasion chambers. In agreement with our observations in

**Figure 2.** Gamma-secretase inhibitor (GSI) treatment reduced AICD and GFAP expression levels in the peri-infarct area. A, GFAP-positive cells and RC2-positive cells expressing AICD and NICD1. Nuclei were stained by DAPI. Scale bars indicate 50 μm. B, Time schedule of treatment. At the time of surgery, GSI or DMSO (vehicle) was injected. Mice were euthanized at 24 hours or 72 hours after treatment. C, AICD expression levels increased in the peri-infarct area 1 day after stroke. In contrast, GFAP expression levels decreased after GSI treatment compared with DMSO treatment (n/H11005 10 to 12 mice for each group). *P/H11021<0.05 compared with DMSO treatment. **P<0.01 compared with sham group. ***P<0.01 compared with DMSO treatment. AICD indicates APP Intracellular Domain; GFAP, glial fibrillary acidic protein; NICD1, Notch 1 Intracellular Domain; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; MCAO, middle cerebral artery occlusion.
vivo, astrocytes cultured in 5% fetal bovine serum significantly decreased Jurkat cell invasion compared with positive migration control (5% fetal bovine serum medium alone; \( n=4 \), \( P<0.05 \); Figure 4B). Assays of lactate dehydrogenase activity showed that astrocyte conditioned medium did not induce cell death in Jurkat cells (\( n=4 \), \( P=0.96 \); Figure 4B).

To understand the effects of \( \gamma \)-secretase-mediated signaling in cultured astrocytes, invasion studies were performed using GSI-treated astrocytes and DMSO-treated astrocytes. GSI treatment significantly reduced the proliferation of astrocytes compared with DMSO treatment (\( n=4 \), Cyquant assay, Day 4, \( P<0.01 \); Day 6, \( P<0.05 \); 5-bromodeoxyuridine incorporation assay, \( n=4 \), \( P<0.05 \); Figure 4C). Assays of lactate dehydrogenase activity showed that GSI treatment did not induce cell death in astrocytes (\( n=3 \), \( P=0.25 \); Figure 4C). After GSI treatment of astrocytes, the number of invading Jurkat cells increased compared with DMSO treatment (\( n=4 \), \( P<0.05 \); Figure 4C).

To determine whether NICD1 levels affect astrocyte proliferation, we transfected cultured astrocytes with NICD1-F2A-green fluorescent protein (GFP) or GFP control plasmid. Two days after transfection, we examined the number of proliferating astrocytes by immunocytochemistry for Ki67. We observed significantly more proliferative astrocytes after NICD1-F2A-GFP transfection compared with GFP transfection alone (\( n=6 \), \( P<0.05 \); Figure 4D).

**Figure 3.** The number of proliferating and RC2-positive reactive astrocytes was significantly decreased after GSI treatment. A. The number of Ki67- and GFAP-positive cells was decreased after GSI treatment compared with vehicle treatment 3 days after stroke (\( n=4 \) mice for each group). B. The number of RC2-positive reactive astrocytes was decreased after GSI treatment 3 days after stroke (\( n=3 \) mice for each group). C. CD45-positive cells in the infarct area were surrounded by RC2-reactive astrocytes. The number of CD45-positive cells that invaded into the peri-infarct area was significantly increased after GSI treatment (\( n=4 \) to 5 mice for each group). Arrows: Double-positive cells. Note: Dotted lines indicate border of stroke infarct core. Scale bars indicate 100 \( \mu \)m. * \( P<0.05 \). GSI indicates \( \gamma \)-secretase inhibitor; GFAP, glial fibrillary acidic protein.

**Notch 1 Inhibition in GFAP-CreERT\(^{TM}\):Notch 1 cKO Mice Reduces Reactive Astrocyte Formation**

To determine whether Notch 1 regulates reactive astrocyte formation in vivo after stroke, we generated GFAP-CreERT\(^{TM}\):tdRFP mice (GR mice) and GFAP-CreERT\(^{TM}\):Notch 1 conditional knockout (cKO) mice (GN cKO mice). In GR mice, tdRFP should be expressed exclusively in GFAP-positive reactive astrocytes after tamoxifen (TM) treatment. In GN cKO mice, Notch 1 should be knocked out exclusively in GFAP-positive reactive astrocytes after TM treatment (Figure 5). First, to confirm the expression patterns of tdRFP in reactive astrocytes after stroke, we administered TM for 3 consecutive days and performed distal middle cerebral artery occlusion surgery 7 days after the last TM administration. Three days after distal middle cerebral artery occlusion surgery, reactive astrocytes were analyzed by immunohistochemistry. In GR mice, 70% of GFAP-positive reactive astrocytes expressed tdRFP (Figure 6A). To confirm that NICD1 was no longer expressed in GFAP-positive cells after TM treatment of GN cKO mice, immunohistochemistry against NICD1 was performed. As expected, after TM treatment, but not after corn oil treatment, NICD1 was not expressed in GFAP-positive cells (Figure 6A). Quantifying GFAP-positive cells, we observed a significantly reduced number of proliferative reactive astrocytes in GN cKO mice after TM treatment (\( n=4 \), \( P<0.01 \); Figure 6B). Notably, the number of RC2-positive reactive astrocytes was also significantly decreased after TM treatment (\( n=4 \), \( P<0.01 \); Figure 6C).
These data demonstrated that Notch 1 plays an important role in reactive astrocyte formation in the peri-infarct area after stroke. To determine whether the decreased number of proliferating reactive astrocytes would affect immune cell invasion, we quantified CD45-positive cell invasion into the peri-infarct area. In agreement with our pharmacological GSI treatment data and ex vivo invasion studies, we also observed a significantly increased number of CD45-positive cells in the peri-infarct area in TM-treated mice (n=4 to 5, P<0.05; Figure 6C). These data showed that proliferating reactive astrocytes, which include RC2-positive reactive astrocytes, are important for suppressing immune cell invasion after stroke.

Discussion

Elucidation of the signaling mechanism(s) that regulate reactive astrocyte formation is important to understand and to treat central nervous system injury. Gamma-secretase-mediated Notch signaling occurs through a conserved pathway that is important for stem cell proliferation and differentiation.16 We found that reactive astrocytes expressed the γ-secretase cleavage products, NICD1 and AICD, in the peri-infarct area after stroke. To examine the effects of γ-secretase activity and Notch 1 signaling on reactive astrocyte formation, we treated mice with GSI and generated GN cKO mice to exclusively knockout Notch 1 in GFAP-positive cells. The number of proliferating reactive astrocytes and RC2-positive reactive astrocytes were both significantly decreased after GSI treatment and also in GN cKO mice, demonstrating that Notch 1 plays a critical role in reactive astrocyte formation after stroke.

Reactive astrocytes were previously shown to prevent immune cell invasion and to reduce inflammation after brain stab injury and spinal cord injury.2,13 We found that the number of invading CD45-positive cells was increased in the
peri-infarct area after GSI treatment and also in GN cKO mice. These data demonstrated that reactive astrocyte proliferation requires Notch 1 and that proliferating reactive astrocytes may have a specialized role in protecting the brain after stroke by decreasing immune cell invasion.

We did not observe an infarct size difference at 3 days after distal middle cerebral artery occlusion in TM-treated GN cKO mice compared with controls, suggesting that Notch 1 knockout in reactive astrocytes may not alter neural protection early after stroke (I.S.S. and J.L.S., unpublished data).

Figure 5. Diagram explaining inducible conditional knock out mouse model.

Figure 6. Knockout of Notch1 in GFAP-CreER<sup>TM</sup>:Notch1 cKO mice (GN cKO mice) reduces reactive astrocyte proliferation. A, tdRFP was expressed in GFAP-positive cells in GFAP-CreER<sup>TM</sup>:tdRFP mice. NICD1 was not expressed in GFAP-positive reactive astrocytes after TM treatment compared with corn oil treatment in GN cKO mice. B, The number of Ki67- and GFAP-double positive cells decreased significantly after TM treatment compared with corn oil treatment 3 days after stroke (n=4 mice for each group). C, CD45-positive immune cells in the infarct area were surrounded by RC2-reactive astrocytes. The number of RC2-positive reactive astrocytes decreased significantly after TM treatment and CD45-positive cell invasion increased significantly after TM treatment compared with corn oil treatment 3 days after stroke (n=4 to 5 mice for each group). Scale bars indicate 100 μm. Arrows: Double-positive cells. Arrowheads: NICD1-negative cells. Dotted lines indicate border of stroke infarct core. *P<0.01. GFAP indicates glial fibrillary acidic protein; NICD1, Notch 1 Intracellular Domain; TM, tamoxifen.
Importantly, the effects of Notch signaling in stroke may differ based on the type of stroke, duration after onset of occlusion, cell type, and signaling through particular Notch ligands/receptors. Arumugam et al. reported that GSI treatment decreased infarct size in a reperfusion model of stroke (intraluminal) by reducing neuronal apoptosis and decreasing the immune response.7 Notch 3 knockout mice were reported to have larger stroke volumes than controls after transient middle cerebral artery occlusion.17 Infusion of Delta-like-4, a Notch ligand, did not alter infarct volume after stroke.18 Additional studies will be necessary to fully understand the roles of Notch signaling in stroke and where and when it is beneficial or detrimental to outcome.

We have shown that Notch 1 is a key factor required for reactive astrocyte proliferation in the peri-infarct area after stroke. Our data also indicate that the proliferative pool of reactive astrocytes surrounding the infarct core suppress immune cell invasion into peri-infarct tissues. Proliferating reactive astrocytes and the RC2-positive subpopulation of immune cell invasion into peri-infarct tissues. Proliferating reactive astrocytes and the RC2-positive subpopulation of reactive astrocytes adjacent to the infarct core may provide important targets for treatment of stroke.

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Disclosures

None.

References

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Mouse

All animal work was approved by the University of Vermont College of Medicine’s Office of Animal Care in accordance with American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. Adult male C57/BL6 mice (6-8 weeks of age) were obtained from Taconic Farms, Hudson, N.Y. We obtained GFAP-CreERTM mice from Dr. Suzanne Baker (St. Jude Children Hospital, Memphis, TN)1. The GFAP-CreERTM mice express CreERTM under control of the 2.2 kb human GFAP (hGFAP) promoter. CreERTM is an inducible form of Cre recombinase that is activated after tamoxifen administration. Tamoxifen binds to CreERTM, resulting in its translocation to the nucleus. We obtained ROSA26-tdRFP mice from Dr. Hans Joerg Fehling (University Clinics Ulm, Germany)2. Due to a loxP-flanked stop codon, the mice do not express tdRed Fluorescent Protein (tdRFP) without exposure to Cre recombinase. ROSA-tdRFP mice were bred to GFAP-CreERTM mice to generate GFAP-CreERTM::tdRFP mice. In GFAP-CreERTM::tdRFP mice, the ubiquitous ROSA promoter produced permanent tdRFP expression in the progeny of Cre-positive cells following tamoxifen administration (in GFAP-positive cells). To delete Notch 1 exclusively in GFAP-positive reactive astrocytes, Notch 1-loxP mice (Jackson laboratory, ME) were bred to GFAP-CreERTM mice. GFAP-CreERTM::Notch 1 mice lose Notch 1 protein exclusively in GFAP-positive reactive astrocytes following tamoxifen administration.

Distal Permanent Middle Cerebral Artery Occlusion (dMCAO).

Mice were anesthetized with isoflurane (1-5 %; Webster Veterinary, Sterling, MA) and body temperature was maintained with a heated pad. Focal cerebral ischemia was produced by permanently occluding the middle cerebral artery (MCA)3-5. Under low-power magnification, the left temporal-parietal region of the head was shaved and an incision was made between the left orbit and the left ear. The MCA was observed through the semitranslucent skull. A small burr hole (1-2 mm) was drilled into the outer surface of the skull just over the MCA. The skull was removed with fine forceps, and the dura was opened with a cruciate incision. The MCA was then encircled with 10-0 monofilament nylon suture (7V33, S & T, Neuhausen, Switzerland), ligated and transected superior to the ligation point. As a sham surgery, mice underwent the same surgery, but not ligation. Gamma-secretase inhibitor (GSI), DBZ (Dibenzazepine, 2 mg/kg in 5 μl of DMSO added to 195 μl PBS; Calbiochem, Darmstadt, Germany) was injected into the left ventricle lumen of the heart under isoflurane anesthesia immediately after dMCAO (200 μl total). As a negative control, we injected the same volume of DMSO in PBS (5 μl of DMSO added to 195 μl of PBS) into mice by intracardiac injection.
Tamoxifen treatment schedule
Twenty mg of tamoxifen (T5648, Sigma) was dissolved in 100 μl of ethanol and 900 μl of corn oil at 56 °C for 2-3 hours. Tamoxifen was stored in the dark at 4 °C until use, up to 3 weeks. Tamoxifen was warmed to 37 °C before administration. GFAP-CreER™:tdRFP mice and GFAP-CreERTM::Notch 1 cKO mice received 2 mg of tamoxifen per 20 g of body weight by intraperitoneal injection once per day for 3 consecutive days starting 10 days before the dMCAO surgery. The dMCAO surgery was performed 7 days after the last tamoxifen administration to ensure that tamoxifen had been eliminated.

Western blotting
Three days following dMCAO, the brain was removed, placed into a polyacrylic brain block, and cut coronally into 1 mm sections. The cortical peri-infarct area was dissected under a light microscope. Total proteins were extracted from the cortical peri-infarct area using a cell lysis buffer (0.1 % SDS, complete protein inhibitor cocktail [Roche, Basel, Switzerland] in PBS). Protein concentrations were determined with a Bio-Rad DC Protein Assay (Bio-rad, Hercules, CA). Protein preparations were separated by SDS-PAGE and transferred to an immobilon-P membrane (PVDF, Millipore, Boston, MA). The membrane was blocked with 5 % non-fat milk and then incubated with a primary antibody. After washing, the membrane was incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature. The blot was visualized by enhanced chemiluminescence and exposed to Kodak X-ray film. Equivalence of protein loading was verified by probing for beta-actin. The antibodies used were against GFAP (Z0334, DAKO, Glostrup, Denmark), AICD (APP Intracellular Domain, 9148-00, Covance), NICD 1 (Notch 1 Intracellular Domain, ab8925, Abcam), Nestin (PRB-315C, Covance) and Beta-actin (A5441, Sigma).

Immunohistochemistry
Three days following dMCAO, mice were euthanized. After perfusion fixation, the brains were post-fixed in 4 % paraformaldehyde overnight and equilibrated in 30 % sucrose at 4 °C. The brains were then frozen in OCT compound and sectioned coronally at 20-30 μm. Frozen sections were treated for immunohistochemical detection: GFAP (1:1000, Z0334, DAKO, Glostrup, Denmark), GFAP (1:500, G3893, Sigma), RC2 (1:20, Hybridoma Bank, University of Iowa, Iowa City, IA), dsred (1:250, 632496, Clontech), Ki67 (1:100, ab1667, Abcam), CD45 (1:1000, CBL1326, Chemicon), AICD (1:500, 9148-00, Covance) and NICD 1 (1:250, ab8925, Abcam). Sections were incubated in 100 % cold methanol for 30 minutes and PBS for 15 minutes. For AICD staining, sections were soaked in 2X SSC (Saline-Sodium Citrate) and microwaved for 10 minutes. Sections were then blocked in blocking buffer (5 % normal goat serum, 0.4 % Triton X in PBS) for 1 hour at room temperature. Sections were incubated in primary antibodies at 4 °C overnight. After 3 X PBS washes, sections were incubated in secondary antisera (Alexa Fluor 488, Alexa Fluor 350 or Alexa Fluor 594, 1:500; Molecular Probes) for 1 hour at room temperature. Isotype controls, rabbit IgG (2 μg/ml), mouse IgG (2 μg/ml), and mouse IgM (2 μg/ml) were used as negative controls for immunohistochemical stains (data not shown, see also Shimada et al.,
For visualization, sections were stained with DAPI (Vector Laboratories, Burlingame, CA) to localize nuclei and were cover-slipped for observation. Photomicrographs were obtained using an epifluorescence deconvolution microscope with an x,y,z stage (Leica DM6000B, Leica, Wetzlar, Germany) with Leica FW4000 software. For deconvolution, images were obtained every 0.1 µm under 100 X magnification. Images were de-convolved using Leica Deblur software.

**Astrocyte isolation**
Primary astroglial cultures were prepared from C57/Bl6 mice (P 0-7). Mouse pups were decapitated and cerebral cortices were dissected and mechanically dissociated in enzyme solution (1.5 mM CaCl₂ [Sigma], 1.5 mM EDTA [Fisher], 2 mg L-Cysteine [Sigma], 20 µg/ml DNase [Worthington Biochemical, Lakewood, NJ], and 200 units papain [Worthington Biochemical] in DMEM: F12 [Gibco]). We plated cells at 5,000 cells/cm². Plates were disturbed by shaking 3 times on the same day as plating to lift off other cell types. Cells were grown in astrocyte growth medium [DMEM:F12 with 10 % FBS, 10 % Glucose (Sigma), 50 units/ml penicillin, and 50 µg/ml streptomycin (Mediatech Inc.)] with medium changes every 2-3 days. In order to confirm the purity of astrocytes, we stained cells and cell lysates with an antibody against GFAP for immunocytochemistry and western blotting, respectively.

**TUNEL assay**
TUNNEL assay (ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit, S7111, Chemicon) was performed according to the manufacturer’s instructions. For combined TUNEL/immunohistochemistry, immunohistochemistry was performed immediately after TUNEL assay.

**Invasion assays**
Astrocytes were plated and cultured for 3 days in 5% astrocyte growth medium (75,000 cells/well in 24 well BD Falcon plates). The growth medium was then removed and 0.75 ml of fresh 5% serum-containing medium was added to begin an invasion experiment. As a positive control, the same volume of fresh 5% serum-containing medium was added to different wells. Two million Jurkat cells were labeled by 2 µM Cell tracker dye (Cell tracker Probes for Long-Term Tracing of Living Cells, C7025, Invitrogen) for 30 minutes at 37 °C. The cells were washed with DMEM:F12 for 30 minutes at 37 °C. Rehydrated inserts were placed into the wells of the 24 well plate and 0.5 ml of DMEM containing 75,000 labeled Jurkat cells was placed into the inserts (BD BioCoat Matrigel Invasion Chambers, 8.0 µm pore size, 354480, BD Falcon). Cell invasion across the insert was assayed 24 hours later with a fluorescence plate reader (Synergy HT, BioTek, Winoski, VT). To study the effects of GSI on astrocytes, astrocytes were treated with DAPT (5µM, D5942, Sigma) for 2 days in the 24 well plate (starting density, 5,000 cells/cm²). Astrocytes were washed with 3 times with PBS to eliminate GSI from the medium and then invasion studies were performed. To determine the effects of GSI on astrocyte proliferation, cell proliferation assays were performed on day 0, 2, 4 and 6 (CyQUANT Cell Proliferation Assay Kit, MP07026, Invitrogen).
**BrDU assay**
Cells were treated with 5µM of BrDU (B5002, Sigma) for 3 days with DMSO or DAPT in 24 well plates. Medium was changed every day. After 3 days, cells were fixed with 4 % paraformaldehyde and stained for BrDU (1:500, AB6326, Abcam). For BrdU staining, cells were treated with 2 N HCl for 30 minutes at 37°C and washed before the incubation with blocking buffer. Staining intensity was measured with a Synergy HT fluorescence plate reader.

**Production of astrocyte conditioned medium**
Astrocytes were cultured in astrocyte growth medium until 90% confluence was achieved in 150 cm² dishes. At these densities, the cells were washed twice with phosphate-buffered saline, and the medium was switched to serum-free alpha minimum essential medium. After 48 hours of incubation, the conditioned medium was collected, filtered (0.2 μm polyethersulfone membrane, NALGENE MF75; Thermo Fisher Scientific), and frozen at –80 °C.

**LDH assay**
To study the effects of astrocyte conditioned medium, Jurkat cells were treated with 5% FBS with astrocyte conditioned medium or alpha MEM for 2 days in the 96 well plate (starting density, 20,000 cells/well). To study the effects of GSI on astrocytes, astrocytes were treated with DAPT (5µM, D5942, Sigma) for 2 days in the 24 well plate (starting density, 5,000 cells/cm²). The medium were collected in a tube and centrifuge at 500G for 4 minutes to remove cells. Supernatant were used for LDH assay according to the manufacture’s protocol (In vitro toxicology assay kit, Lactid Dehydrogenase based, TOX7, Sigma).

**NICD 1 overexpression**
As a source of NICD 1 coding sequence we used the retroviral vector pCLEN (Addgene plasmid 17704)⁶. We cloned NICD coding sequence into lentiviral vector pWPT-GFP (Addgene plasmid12255). To maximize an expression of downstream located GFP we generated transcriptional/translational fusion between NICD 1 and GFP sequences with F2A peptide sequence as a linker using overlap extension PCR method⁷. This peptide was originally discovered as a key factor of co-expression of multiple viral genes resulting in production of polyprotein followed by self-cleavage within conservative aminoacid motif of the peptide and formation of individual proteins⁸. PCR product was cloned into pCR.2.1–TOPO vector (Invitrogen), verified by sequencing and inserted into BamH1, and Sal1 sites of the pWPT lentivirus vector. Plasmid transfection on astrocytes was performed using Lipofectamine Reagent (Lipofectamine 2000 Reagent, 11668-027, Invitrogen). Astrocytes were plated in 24 well plates at starting density 5,000 cells/cm². The next day, 0.2 µg of DNA, 0.5 µl of Lipofectamine LTX and 40 µl of alpha-MEM were mixed and incubated for 25 minutes at room temperature. Mixed solution was added to cultured astrocytes and incubated for 1 day. Astrocytes were fixed with 4 % paraformaldehyde in PBS. Immunocytochemistry was performed to detect Ki67-positive proliferating cells. The number of Ki67-positive cells and GFP-positive cells were quantified in a whole field of 24 well plates under a microscope.
Cell quantification and statistics
Infarct area was defined by tissue autofluorescence, while peri-infarct area was defined by the presence of RC2- or GFAP-positive reactive astrocytes. One out of every ten sections was quantified. Cell counts were performed by observers blinded to slide (sample) identity. Cell numbers were counted using Fractionator Probe (cells/cm$^2$) or Optical Fractionator (cells/cm$^3$) of Stereo Investigator in the cortical peri-infarct area (MBF Biosciences, Williston, VT). Stereo Investigator was used in conjunction with a Nikon Optiphot 2 microscope, a Hitachi HVC20 camera, a Heidenhahn focus encoder, and a motorized, computer-driven X–Z stage (all microscope attachments provided by MBF Biosciences). Under low magnification, the area of interest was identified and the boundary contour was drawn using the software-pointing device. Spacing between sampling sites (grid size) was set such that 6-12 sampling sites were counted per section. For RC2-positive and GFAP-positive cell quantification, cell identity was ascertained by DAPI localization. At 3 days after stroke, RC2-positive reactive astrocytes were located in the area that covered 200 µm radially outside the edge of the infarct core. We therefore defined the inner peri-infarct area as cortical tissue that covered 200 µm radially outside the edge of the infarct core where RC2-positive cells were typically located. We defined the outer peri-infarct area as the cortical tissue that covered 200 µm radially outside of the inner peri-infarct area. All data were expressed as means and standard error means. Comparisons made between any two groups were performed by Student's t-test. Values of P < 0.05 were considered significant.

SUPPLEMENTAL REFERENCES

5. Shimada IS, Peterson BM, Spees JL. Isolation of locally derived stem/progenitor cells from the peri-infarct area that do not migrate from the lateral ventricle after cortical stroke. *Stroke*. 2010